Metabolic Engineering of Ammonium Assimilation in Xylose-Fermenting Saccharomyces cerevisiae Improves Ethanol Production

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Received 28 February 2003/Accepted 2 June 2003

Cofactor imbalance impedes xylose assimilation in Saccharomyces cerevisiae that has been metabolically engineered for xylose utilization. To improve cofactor use, we modified ammonia assimilation in recombinant S. cerevisiae by deleting GDH1, which encodes an NADPH-dependent glutamate dehydrogenase, and by overexpressing either GDH2, which encodes an NADH-dependent glutamate dehydrogenase, or GLT1 and GLN1, which encode the GS-GOGAT complex. Overexpression of GDH2 increased ethanol yield from 0.43 to 0.51 mol of carbon (Cmol) Cmol⁻¹, mainly by reducing xylitol excretion by 44%. Overexpression of the GS-GOGAT complex did not improve conversion of xylose to ethanol during batch cultivation, but it increased ethanol yield by 16% in carbon-limited continuous cultivation at a low dilution rate.

In order to develop an efficient process for the production of bioethanol from lignocellulosic material, there have been many attempts to improve the conversion of xylose to ethanol by construction of recombinant Saccharomyces cerevisiae strains. Even though the open reading frames encoding the three first enzymes involved in xylose metabolism, i.e., xylose reductase (XR), xylitol dehydrogenase (XDH), and xylulose kinase (XX), have been identified in the genome of S. cerevisiae, xylitol dehydrogenase 1, encoded by XDH, is NADH dependent and is encoded by XDH1. The other glutamate dehydrogenase that is present in S. cerevisiae is NADH dependent and is encoded by GDH2. This enzyme is usually catalyzing the degradation of glutamate into 2-ketoglutarate and ammonium (8). Coaction of two other enzymes, glutamate synthase (GLT1) and glutamine synthetase (GLN1), known as the GS-GOGAT system, may also assimilate ammonia into glutamate, using NADH as a cofactor and ATP. Nissen et al. (9) have shown that deletion of GDH1 in S. cerevisiae resulted in an increased ethanol yield, concomitant with a decreased glycerol yield. This was due to a shift from use of NADPH to use of NADH in connection with ammonia assimilation, and hereby NADH generated in connection with biomass formation could be balanced through ammonia assimilation instead of through formation of glycerol. However, the specific growth rate was dramatically reduced, and overexpression of either GDH2 or the GS-GOGAT system was necessary to recover the growth performance. Since this strategy decreases the utilization of NADPH in ammonia assimilation, more NADPH will be expected to be available for the reduction of xylose to xylitol.

In the present study, we therefore overexpressed GDH2 or GLN1 and GLT1 in a strain with a deletion of gdh1. The XYL1, XYL2, and XKS1 genes were introduced in each of these strains, resulting in xylose-fermenting strains. Batch and chemostat cultivations were carried out in order to investigate the physiology of the recombinant strains and to analyze the effect of redox balance modification on xylose metabolism.

MATERIALS AND METHODS

Strains. All S. cerevisiae strains used in this study were derived from the CEN.PK 113-7D wild-type strain (Table 1). The genes encoding XR and XDH
from P. stipitis and the endogenous gene for XK have already been integrated into the chromosome of CEN.PK 113-7D, using the integrative plasmid YipXR/XDH/XK, leading to the stable construct TMB3001 (3). The GDH1 gene has been deleted using the loxP-kanMX-loxP disruption cassette (5). GDH1, GLN1, and GLT1 have been put under a PGK constitutive promoter. Transformation with the plasmid YipXR/XDH/XK was performed using the lithium acetate method as described by Gietz et al. (4). The strains were stored at 4°C on yeast extract-peptone-dextrose agar plates.

**Media preparation.** A defined medium containing trace metal elements and vitamins was used in all cultivations. Fatty acids in the form of Tween 80 and ergosterol were added to the cultivations to sustain anaerobic growth of *S. cerevisiae*. The medium used for batch and chemostat cultivations was prepared according to the method of Verduyn et al. (18). For cultivation on glucose, the concentration was 20 g liter⁻¹; for mixed sugar cultivations, 20 g of glucose liter⁻¹ and 50 g of xylose liter⁻¹ were used.

**Batch cultivation.** Cultivations were carried out in well-controlled four-baffled 5-liter in-house-manufactured bioreactors with a working volume of 4 liters, and the temperature was controlled at 30°C. The bioreactors were equipped with two disk-turbine impellers rotating at 500 rpm. The pH was kept constant at 5.0 by automatic addition of 2 M NaOH. Nitrogen containing less than 5 ppm O₂ (AGA, Copenhagen, Denmark) was used for sparging of the fermentor at 0.2 vvm. Batch cultivations were performed in duplicate or triplicate in order to evaluate the reproducibility of the experiments. A mean value was calculated for all the parameters, as well as the standard deviation.

**Continuous cultivation.** Carbon-limited cultivations were carried out in 2-liter Applikon (Schiedam, The Netherlands) bioreactors with a constant working volume of 1 liter at 30°C and with a stirring speed of 500 rpm. The pH was kept constant at 5.0 by automatic addition of 1 M NaOH. Nitrogen containing less than 5 ppm of O₂ (AGA, Copenhagen, Denmark) was used for sparging of the fermentor at 0.2 vvm. Batch cultivations, chemostat cultivations were performed in duplicate or triplicate in order to evaluate the reproducibility of the experiments.

**Off-gas analysis.** Carbon dioxide and oxygen concentrations in the exhaust gas were determined by a Bruel & Kjaer (Nærum, Denmark) 1308 acoustic gas analyzer (2).

**Cell mass determination.** The dry weight was determined as previously described (14). Biomass composition used in yield calculation was CH₁₅O₇N₁₉.H₂O.

**Analysis of extracellular metabolites.** Samples taken from the cultivation broth were immediately filtered through a 0.45-µm pore-size cellulose acetate filter (Osmonics) and stored at −20°C until analysis. Glucose, xylose, xylitol, glycerol, ethanol, succinate, and acetate concentrations were determined as previously described (14).

### RESULTS

**Batch cultivation.** Physiology of xylose-fermenting *S. cerevisiae* strains CPB.CR1 (Δgdh1), CPB.CR4 (Δgdh1 GDH2), and CPB.CR5 (Δgdh1 GS-GOGAT) in comparison with the parental type strain, TMB3001, was investigated during anaerobic batch cultivations on 20 g of glucose liter⁻¹ or a mixture of 20 g of glucose liter⁻¹ and 50 g of xylose liter⁻¹. Deletion of *gdh1* in CPB.CR1 resulted in a decrease in the specific growth rate to 0.16 (±0.002) h⁻¹ compared with 0.34 (±0.006) h⁻¹ for the parental strain TMB3001 during batch cultivation on glucose (Table 2). Overexpression of GDH2 or the GS-GOGAT system in the *gdh1* deletion background resulted in a recovery of the specific growth rate to 0.32 (±0.002) h⁻¹. Deletion of *gdh1* led to a 48% decrease in the glycerol yield during batch cultivation on glucose down to 0.057 (±0.001) mol of carbon (Cmol Cmol⁻¹). Similarly, the glycerol yield in CPB.CR4 and CPB.CR5 decreased to 0.06 (±0.004) and 0.07 (±0.001) Cmol Cmol⁻¹, respectively, compared to 0.11 (±0.003) Cmol Cmol⁻¹ in TMB3001. Nevertheless, *gdh1* deletion alone did not improve ethanol yield, but it decreased to 0.41 (±0.006) Cmol Cmol⁻¹ compared to 0.53 (±0.01) Cmol Cmol⁻¹ for the parental type. For CPB.CR4, the increase in ethanol yield (0.56 ± 0.01 Cmol Cmol⁻¹) was 5%, whereas for CPB.CR5 the increase was not significant compared to the level in the parental strain (Table 2).

When cells were grown on a mixture of glucose and xylose, the glucose consumption rate decreased compared with growth on glucose alone (Table 2). Similarly, the specific growth rates were generally lower on the sugar mixture than with glucose as the sole carbon source. In CPB.CR1 as well as in CPB.CR5, the total sugar consumption rate decreased to 86 (±6.2) to 88 (±8.4) Cmol of sugar g (dry weight)⁻¹ h⁻¹ compared to 149 (±11) Cmol of sugar g (dry weight)⁻¹ h⁻¹ in TMB3001. On the contrary, overexpression of GDH2 (CPB.CR4) increased the total sugar consumption rate by 20% up to 180 (±12) Cmol g (dry weight)⁻¹ h⁻¹. As illustrated in Fig. 1, xylose was consumed much faster by CPB.CR4 than by TMB3001 in the initial phase of xylose consumption. We calculated the maximum xylose consumption rate at the time of glucose depletion. It increased from 13 (±0.6) in TMB3001 to 15 (±0.9) Cmol g (dry weight)⁻¹ h⁻¹ in CPB.CR4 (Table 2). After glucose depletion, the specific xylose consumption rate slowly decreased, and no difference of the xylose consumption rate could then be observed between the two strains after 100 h. Overexpression of the GS-GOGAT complex did not affect the maximum rate of xylose consumption during batch cultivation on a mixture of glucose and xylose. The glycerol yield was higher in CPB.CR1 (0.13 ± 0.005 Cmol Cmol⁻¹) and CPB.CR4 (0.10 ± 0.002 Cmol Cmol⁻¹) than was lower in CPB.CR5 (0.05 ± 0.001 Cmol Cmol⁻¹) than the parental strain, TMB3001 (0.07 ± 0.001 Cmol Cmol⁻¹) (Table 2). An 18% increase in the ethanol yield was achieved during batch cultivation of glucose and xylose by overexpression of GDH2 and deletion of *GDH1* in CPB.CR4 from 0.43 (±0.01) to 0.51 (±0.01) Cmol Cmol⁻¹, with a concomitant decrease in the xylitol yield from 0.29 (±0.006) Cmol Cmol⁻¹ down to 0.16 (±0.005) Cmol Cmol⁻¹, whereas the two other constructs had lower ethanol yields than the parental strain (Table 2).

**Chemostat cultivation.** Chemostat cultivations of the metabolically engineered strains were performed in order to analyze the metabolism of the strains at low sugar consumption rates, with the dilution rate set to 0.05 h⁻¹, i.e., the specific growth rate was 0.05 h⁻¹. The carbon source was either 20 g of glucose liter⁻¹ or a mixture of 20 g of glucose liter⁻¹ and 50 g of xylose liter⁻¹ as during batch cultivation. As for the batch cultivation,
glycerol yield on glucose decreased from 0.07 (±0.001) Cmol Cmol⁻¹ in TMB3001 down to 0.05 (±0.002), 0.06 (±0.001), and 0.05 (±0.001) Cmol Cmol⁻¹ in CPB.CR1, CPB.CR4, and CPB.CR5, respectively. The ethanol yield was improved in all the redox engineered strains, where the ethanol yield increased maximally with 14% in CPB.CR1 to 0.58 (±0.009) Cmol Cmol⁻¹, compared to 0.51 (±0.012) Cmol Cmol⁻¹ in the parental strain (Table 3).

When cells were grown on a mixture of glucose and xylose, they all presented a biomass yield in the range of 0.07 to 0.09 Cmol Cmol⁻¹ (Table 3). The glycerol yield was lower in CPB.CR1 and CPB.CR4, 0.02 (±0.001) and 0.03 (±0.001) Cmol Cmol⁻¹, respectively, whereas it was higher, 0.08 (±0.002) Cmol Cmol⁻¹ in CPB.CR5, compared to 0.06 (±0.001) Cmol Cmol⁻¹ in TMB3001. The ethanol yield during growth on the glucose-xylose mixture was higher in the three redox engineered strains, i.e., 0.40 (±0.01), 0.44 (±0.02), and 0.43 (±0.023) Cmol Cmol⁻¹ in CPB.CR1, CPB.CR4, and CPB.CR5, respectively, compared to 0.37 (±0.015) Cmol Cmol⁻¹ in the parental strain. Consequently, the xyitol yield was lower, 0.22 (±0.006), 0.19 (±0.005) and 0.17 (±0.01) Cmol Cmol⁻¹ for CPB.CR1, CPB.CR4, and CPB.CR5, respectively. Nevertheless, the xylose consumption rate was lower for the gdh1 deletion strain than for TMB3001. Overexpression of GDH2 resulted in an increased xylose consumption rate compared to that of the gdh1 deletion strain, but the specific xylose consumption rate was the same in TMB3001 and CPB.CR4, 10.2 (±0.51) and 10.3 (±0.41) Cmmol g (dry weight)⁻¹ h⁻¹, respectively. However, contrary to what was found during the batch cultivations, overexpression of the GS-GOGAT system in a gdh1 deletion background enhanced the xylose consumption rate from 10.2 (±0.51) to 11.4 (±0.52) Cmmol g (dry weight)⁻¹ h⁻¹ (Table 3).

DISCUSSION

In this study, we showed that metabolic engineering of the ammonia assimilation in xylose-fermenting S. cerevisiae resulted in a substantially improved conversion of xylose to ethanol during anaerobic batch growth on glucose and xylose mixture and particularly that the formation of the by-product xyitol could be reduced by 44% in S. cerevisiae CPB.CR4 (Table 2). From analysis of the different recombinant strains in batch and chemostat cultures, the different results obtained between the engineered strains could basically be explained by two effects: (i) the modification of the redox metabolism in the recombinant strains and (ii) an increased use of ATP in the recombinant strain CPB.CR5 with overexpression of the GS-GOGAT pathway.

In the S. cerevisiae CPB.CR4 strain, NADH is used in ammonia assimilation due to the overexpression of GDH2, resulting in an improved availability of NAD⁺ for the XDH in the xylose metabolism. Hereby, there is an increased conversion of xyitol to xylulose (44% lower xyitol yield than that of the parent strain). Furthermore, with the shift in cofactor use in the ammonia assimilation, NADPH is no longer used for glutamate synthesis and can be dedicated for use by the XR in the conversion of xylose to xyitol. Thus, xylose conversion was favored, and the consumption rate consequently increased by 15% compared to that of the parental strain TMB3001.

For CPB.CR5 with overexpression of the GS-GOGAT complex, there was an increased drain of ATP for ammonia assimilation. Since 1 mol of ATP is used to convert 1 mol of 2-ke-togluartate by the GS-GOGAT system, the ATP demand for growth is higher in CPB.CR5 than in CPB.CR1 and CPB.CR4, where only the cofactor use was modified. The intracellular ATP concentration has been reported in an S. cerevisiae strain to be as low as 1.78 mM where XK has been overexpressed during anaerobic fed-batch cultivation on xylose (17). These cultivation conditions are close to the ones prevailing during the batch cultivation of CPB.CR5. Therefore, one can assume that intracellular ATP concentration in CPB.CR5 is below 1.78 mM, since both XK and GS-GOGAT are overexpressed, and even below the affinity of ATP for XK, which has been reported to be around 1.5 mM (11). Hence, XK cannot operate efficiently, and xylose phosphorylation is not sufficient to
make the downstream conversion of xylitol favorable (thermo-
dynamics of XR and XDH favor formation of xylitol) (12, 13).
Consequently, 20% more xylitol was produced than was the
case with TMB3001. During chemostat cultures (Table 3), we
find that in all the redox-engineered strains, ethanol yield from
glucose and xylose was improved. In \textit{S. cerevisiae} CPB.CR5, the
xylose consumption rate increased by 15% and the xylitol ex-
cretion was 25% lower than in the parental strain, TMB3001.
During carbon-limited chemostat cultivations at a low dilution
rate, ATP levels are more elevated than during batch cultiva-
tions (16), and the kinase effect, originating from the coaction
of GS-GOGAT and XK, was thereby lower than during batch
cultivations, explaining why xylose conversion was enhanced in
CPB.CR5.

In conclusion, engineering of ammonium assimilation made
it possible to modify the redox balance in xylose fermenting \textit{S.}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|c|c|}
\hline
Strain & Concn (g/liter) of carbon source(s) & $r_{glc}^{a}$ (Cmol/gDW-h) & $r_{xyl}^{a}$ (Cmol/gDW-h) & $Y_{xslc}^{b}$ (Cmol/Cmol) & $Y_{xyl}^{b}$ (Cmol/Cmol) & $Y_{xyl}^{c}$ (Cmol/Cmol) & $Y_{xyl}^{d}$ (Cmol/Cmol) & $Y_{xyl}^{e}$ (Cmol/Cmol) & $Y_{xyl}^{f}$ (Cmol/Cmol) \\
\hline
TMB3001 & 20 glucose & 19.6 & 0.11 & 0.07 & 0.51 & 0.29 & \\
 & 20 glucose + 50 xylose & 13.9 & 0.08 & 0.06 & 0.37 & 0.27 & 0.23 & 0.55 \\
CPB.CR1 ($\Delta$gdh1) & 20 glucose & 22 & 0.09 & 0.05 & 0.58 & ND & \\
 & 20 glucose + 50 xylose & 15.9 & 0.09 & 0.02 & 0.40 & 0.31 & 0.22 & 0.58 \\
CPB.CR4 ($\Delta$gdh1 GDH2) & 20 glucose & 17.1 & 0.12 & 0.06 & 0.53 & 0.27 & \\
 & 20 glucose + 50 xylose & 15.6 & 0.07 & 0.03 & 0.44 & 0.23 & 0.19 & 0.46 \\
CPB.CR5 ($\Delta$gdh1 GS-GOGAT) & 20 glucose & 16 & 0.12 & 0.055 & 0.52 & 0.28 & \\
 & 20 glucose + 50 xylose & 15 & 0.07 & 0.08 & 0.43 & 0.23 & 0.17 & 0.43 \\
\hline
\end{tabular}
\caption{Specific sugar consumption rates and product yield coefficients for anaerobic carbon-limited chemostat fermentations at a dilution rate of $D = 0.05$ h$^{-1}$.}
\end{table}

\textit{Notes:}

\begin{itemize}
\item $a$ Specific consumption rate of glucose.
\item $b$ Specific consumption rate of xylose.
\item $c$ Yields (Y) are based on total consumed sugars. Yield for biomass.
\item $d$ Yield for glycerol.
\item $e$ Yield for ethanol.
\item $f$ Yield for CO$_2$.
\item $g$ Yield for xylitol.
\item $h$ Xylitol yield based on consumed xylose.
\item ND, not determined.
\end{itemize}
cerevisiae. Previous work on the modification of cofactors utilization resulted either in a decreased xylitol yield concomitant with an alteration of the xylose consumption rate (7) or in an increased xylose consumption rate accompanied with an increased xylitol excretion (1). In this study, it was possible to improve both the xylose consumption rate and the ethanol yield from xylose by deletion of GDH1 and overexpression of either GDH2 or both GLT1 and GLN1. This confirms the importance of balanced cofactor use in xylose metabolism.

ACKNOWLEDGMENTS

Bärbel Hahn-Hägerdal, Department of Applied Microbiology, Lund University, Lund, Sweden, is sincerely thanked for kindly providing the plasmid carrying the XYL1. We are also grateful to Margarida dos Santos, CPB, Biocentrum-DTU, for providing the strains CEN.MS1 and CEN.MS5. Previous work on the modification of cofactors utilization resulted either in a decreased xylitol yield concomitant with an alteration of the xylose consumption rate (7) or in an increased xylose consumption rate accompanied with an increased xylitol excretion (1).

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